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13. ABSTRACT ***(Maximum 200 Words)***

In the present work we have capitalized on the availability in our laboratory of an in vitro model of transformation of immortalized HBEC by the chemical carcinogen BP for comparison with phenotypic and genomic changes induced by the natural estrogen 17b-estradiol (E2). Short term treatment of these cells with physiological doses of 17-b estradiol induces anchorage independent growth, colony formation in agar methocel, and reduced ductulogenic capacity in collagen gel, all phenotypes whose expression is indicative of neoplastic transformation, and that are induce by BP under the same culture conditions. The fact that the MCF10F cells are both ER-a and ERb. negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. We have found that estrogen induces LOH in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2-25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice.

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Estrogens and Breast Cancer

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5-INTRODUCTION

Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones [1-4]. Breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors [5,6], from which estrogens have been demonstrated to be of essential importance in this phenomenon as it is observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity [7,8].

Estrogens, that are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors (ER) α or β. Estrogens might act as well through alternate non-receptor mediated pathways [17]. E_2, under the effect of 17 α -oxidoreductase is continuously interconverted to estrone (E_1), and both are hydroxylated at C-2, C-4, or C-16 α positions by cytochrome P450 isoenzymes, i.e., CYP1A1, CYP1A2, or CYP1B1, to form catechol estrogens [18-23]. The demonstration that the catecholestrogen 4-hydroxyestradiol (4-OH-E_2) induces an estrogenic response in the uterus of ER α null mice, and the fact that this response is not inhibited by the antiestrogen ICI-182, 780 [9], indicate that this catecholestrogen does not exert its effect on the ER. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA via a Michael addition and thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidatic activation of CE. Thus, estrogen and estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis [18-23].]. Although this pathway has been demonstrated in other systems [18-20], it still needs to be demonstrated in human breast epithelial cells.

Furthermore, if estrogen is carcinogenic in the human breast through the above mentioned pathway it would induce in breast epithelial cells in vitro transformation phenotypes indicative of neoplasia and also induce genomic alterations similar to those observed in spontaneous malignancies, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes [24-39]. In order to test this hypothesis we have evaluated the transforming potential of E_2 on human breast epithelial cells (HBEC) in vitro, utilizing the spontaneously immortalized HBEC MCF-10F [40,41]. This cell line lacks both ER- α and ER-β although this latter receptor is induced in cells transformed by chemical carcinogens [42]. In the present work we report that the same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogen benz (a) pyrene (BP) and oncogenes [43-46] were expressed in E_2 treated cells. E_2 transformed cells exhibited loss of heterozygosity (LOH) in loci of chromosome 11, known to be affected in spontaneously occurring breast lesions, such as ductal hyperplasia, carcinoma in situ, and invasive carcinoma [47-60].

6-BODY

Direct mutagenic and transforming activities of estrogens in cultured Syrian hamster embryonic cells were documented more than a decade ago, but whether these results are relevant to human situation has been subject to debate because rodent cells in general are easier to be transformed than human cells. Therefore, we have investigated the carcinogenic potential of

estrogens in human breast epithelial cells in vitro. Using our established in vitro cell transformation model we have proposed tow specific aims 1-To determine whether estrogens induce neoplastic transformation of normal HBEC MCF10Fcells through receptor mediated hormonal activities and 2-To determine whether estrogens promote neoplastic progression of estrogen or chemical carcinogen transformed derivatives of MCF10F cells. **This progress report covers the period of August 1 2000 to July 30, 2001, period in which we have been able to accomplish part of the Specific Aim 1 as indicated below.**

i-Determination of the dose response curve to 17 beta estradiol

In order to determine the optimal doses for the expression of the cell transformation phenotype we treated the immortalized human breast epithelial cells (HBEC) MCF-10 F with 17-β-estradiol (E_2) for testing the survival efficiency (SE) whether they express colony formation in agar methocel, or colony efficiency (CE), and loss of ductulogenesis in collagen matrix. MCF-10F cells were treated with 0.0, 0.07 nM, 70 nM, or 0.25 mM of E_2 twice a week for two weeks. (Figure 1). Based upon these results a dose of 3.7μM (1μg/ml) was selected for testing the effect of E2, progesterone (P), and benzo (a) pyrene (BP).

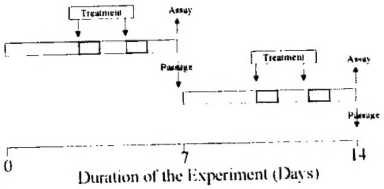

Figure 1. MCF-10F cells were treated with E_2, DES, or BP at 72 hrs and 120 hours post plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis.

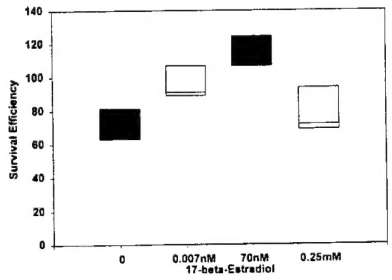

Figure 2: Box plot showing the dose effect of 17-β-estradiol on the survival efficiency in agar methocel of MCF-10F cells.

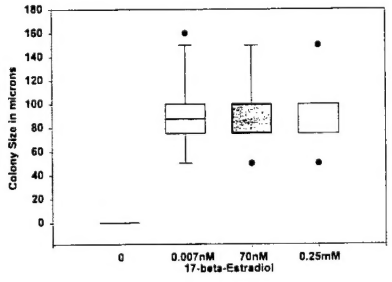

Figure 3: Box plot showing the dose effect of 17-β-estradiol on colony size.

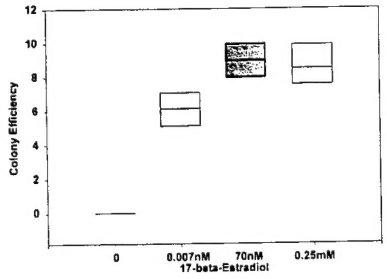

Figure 4: Box plot showing the dose effect of 17-β-estradiol on colony efficiency.

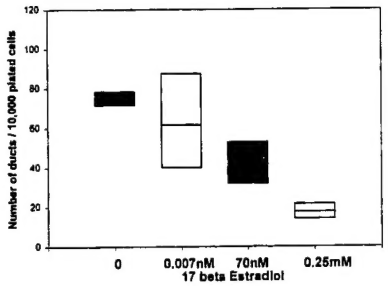

Figure 5: Box plot showing the dose effect of 17- β-estradiol on MCF-10F cells forming ductules in collagen matrix over 10,000 cells plated..

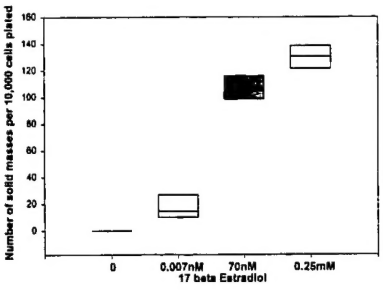

Figure 6: Box plot showing the dose effect of 17- β-estradiol on MCF-10F cells forming solid or spherical masses in collagen matrix per 10,000 cells plated..

The survival efficiency (SE) was increased with 0.007nM and 70 nM of 17 β estradiol and decrease with 0.25mM (Figure 2). The cells treated with either doses of E2 formed colonies in agar methocel and the size was not different among them (Figure 3), however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses (Figure 4). Ductulogenesis or the number of ductules per 10,000 cells plated, was 75±4.9 in control cells; it decreased to 63.7±28.8, 41.3±12.4, and 17.8±5.0 in E_2 treated cells (Figure 5), which also formed spherical like structures or solid masses (Figures 7a-d), whose numbers increased from 0 in controls to 18.5±6.7, 107±11.8 and 130±10.0 for each E_2 dose (Figure 6).

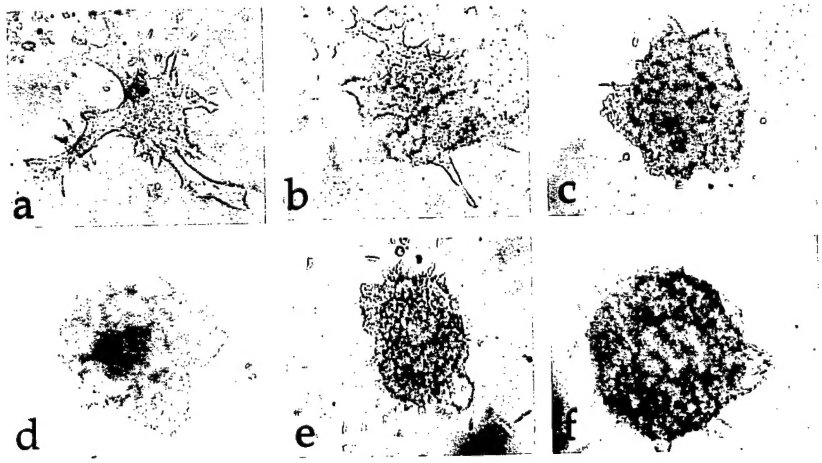

Figure 7a; MCF 10F cells treated with Solvent (DMSO) forming well defined ductular structures in collagen matrix; b, 0.007nM of E2 induces alteration in the ductular pattern; c and d, 70nM of E2 induces the loss of ductular formation in collagen matrix; e and f, 1μg of E2 or BP, respectively, induces the formation of spherical masses in collagen matrix. Phase contrast microscope X10. This in vitro technique evaluates the capacity of cells to differentiate by providing evidence of whether treated cells form tridimensional structures when grown in a collagen matrix. Parental, control, and treated cells were suspended at a final density of 2 $x10^3$ cells/ml in 89.3% $Vitrogen^{100}$ collagen matrix (Collagen Co., Palo Alto, CA) and plated into four 24 well chambers pre-coated with agar base. The cells were fed fresh feeding medium containing 20% horse serum twice a week. The cells were examined under an inverted microscope for a period of 21 days or longer for determining whether they formed ductule-like structures or whether they grew as unorganized clumps. The final structures were photographed, and then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological examination.

ii- ***Effect of estrogen, progesterone and benz(a)pyrene on the expression of transformation phenotypes:***

MCF-10F cells were treated with 1.0 □g/ml E_2 (Aldrich, St. Louis, MO), progesterone (Sigma Chemical Co., St. Louis, MO), control cells were treated with DMSO. MCF-10F cells treated with 1.0 □g/ml benz (a) pyrene (BP) served as positive controls for cell transformation assays. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with E_2, P, or BP at 72 hrs and 120 hours post plating. At the end of the first week of treatment, the cells were passaged for administration of another two periods of hormonal treatment. Treatments were repeated during the second week, and cells were collected at the 14^{th} day for phenotypic and genotypic analysis (Figure 1). At the end of each treatment period the culture medium was replaced with fresh medium. At the end of the second week of treatment the cells assayed for determination of survival efficiency (SE), colony efficiency (CE), colony size (CS), and ductulogenic capacity, as described in previous publications [44,45].

The survival efficiency of MCF-10F cells was increased with all the treatments (Figure 8). Evaluation of colony formation at the end of the second week of E_2 and BP treatment revealed that MCF-10F cells formed colonies in agar-methocel over 60 microns in diameter, whereas those cells treated with progesterone the colonies are smaller (Figure 9). MCF-10F control cells treated with DMSO did not form colonies. (Figure 9). The total colony efficiency (CE) was significantly increased by E2 and BP, and significantly less by Progesterone (Figures 10 and 11a-i)

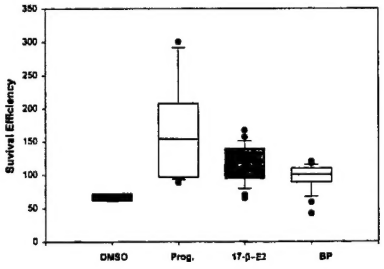

Figure 8: Box plot showing the effect of different compounds on MCF-10F cells survival efficiency in agar methocel.

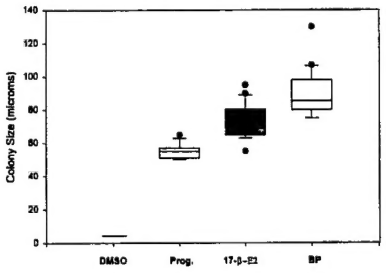

Figure 9: Box plot showing the effect of different compounds on MCF-10F cells colony size growing in agar methocel.

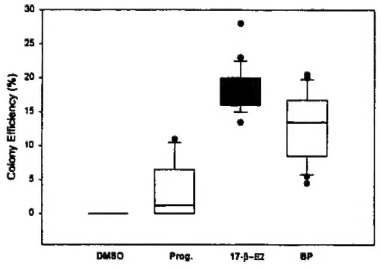

Figure 10: Box plot showing the effect of different compounds on MCF-10F cells colony efficiency in agar methocel.

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form tubular and ductular structures. It was maximal in MCF-10F cells (Figure 6a), and completely negative (-) in BP-treated cells, which grew as a solid or cystic mass. All the cells treated with E_2 exhibited decreased ability to form ductules (Figures 7b-e). Progesterone does not affect significantly the ductulogenic capacity. The collagen matrix

embedded in paraffin and cross sectioned for determination of cell morphology showed that MCF10F form a well defined ductule lined by a monolayer of cuboidal epithelial cells (Figure 12a), whereas those treated with E2 the number of layers increase and in some cases the whole lumen is obliterated (Figures 12b-d). BP also forms similar structures to those induced by estrogen, whereas the ductules formed by progesterone treatment are smaller with a reduced luminal size lined by a monolayer of cuboidal epithelial cells.

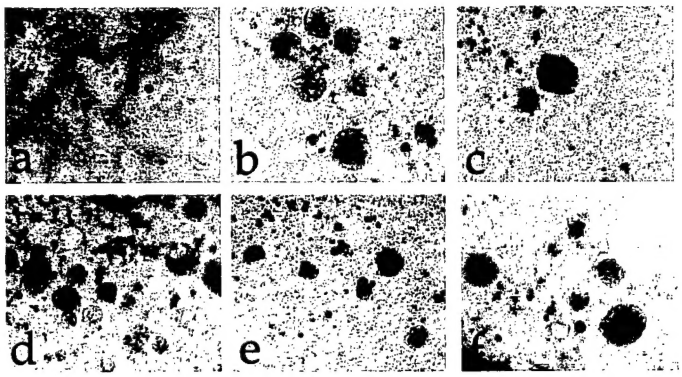

Figure 11. MCF-10F cells plated in agar-methocel for colony assay. (a); control cells do not form colonies, only isolated cells are present; (b-d), colonies formed by E_2-treated MCF-10F cells at the doses of 0.007nM, 70nM and 1μM respectively; (e); Progesterone treated cells; (f), BP-treated cells induces slightly larger colonies. Phase contrast microscopy X 4. This technique was utilized as an in vitro assay for anchorage independent growth, a parameter indicative of transformation. Parental, control, and treated cells were suspended at a density of $2x10^4$ cells/ml in 2 ml of 0.8% methocel (Sigma Co, St. Louis, MO) dissolved in DMEM:F-12 (1:1) medium containing 20% horse serum. Cells from each treatment group and time point were plated in four 24-well chambers pre-coated with 0.5 ml of 0.8% agar base in DMEM: F-12 medium, which was replaced with fresh feeding medium containing 0.8% methocel twice a week. The actual number of cells plated was calculated as the average of cells counted at 10x magnification in 5 individual fields, and multiplied by a factor of 83. CE and CS were measured 21 days after plating. CE was determined by a count of the number of colonies greater than 50 μm in diameter, and expressed as a percentage of the original number of cells plated per well.

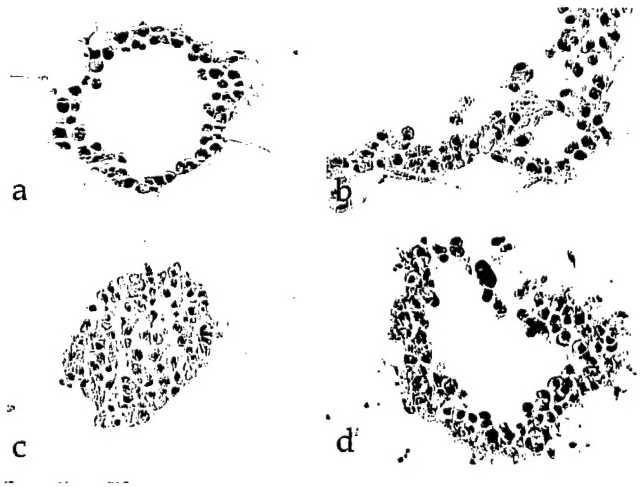

Figure 12. Histological sections of cells growing in collagen gel. The cells have been fixed in buffered formalin, embedded in paraffin and the sections stained with hematoxylin and eosin. a; MCF 10F cells treated with solvent (DMSO) forming well defined ductular structures lined by a single cuboidal layer of cells; b, 0.007nM of E2 induces alteration in the ductular pattern forming spherical masses lined by two to three layers of cells; c, 70nM of E2 induces the loss of ductular formation in collagen matrix and the solid spherical masses are composed of large cuboidal cells; e,1μg of E2 or BP induces the formation of spherical masses lined by multiple layers of cells. Phase contrast microscope X10.

iii-Genomic changes induced in E_2 and DES transformed MCF-10 cells.

From the E2 treated cells six clones out 24 colonies were expanded and maintained in culture. These clones were designated E_2-1 to E_2-6 (Table 1). These clones were selected for genomic analysis. DNA fingerprint analysis of parent, E_2, P, and BP-treated cells and their derived clones revealed that their allelic pattern was identical in all the cell lines analyzed. These results confirmed that all the cells tested had the same HBEC origin, and that they were free of contamination from other cell lines maintained in our laboratory.

TABLE 1: PHENOTYPIC MARKERS OF CELL TRANSFORMATION INDUCED IN MCF-10F CELLS BY 17β ESTRADIOL (E_2), AND BENZ (A) PYRENE (BP)

Cell Type	No. of Passages	Doubling time (DT)[a]	Colony Number (CN)[b]	Colony Efficiency (%)(CE)[c]	Colony Size (CS)(□m)[d]
MCF-10F	113	93±5.6	0.0	0.0	0.0
BP	4	42±3.8	89	18±4.5	670±46
E_2	4	78±16.0	24[e]	4.8±0.9	170±34
E_2-1*	4	81±3.0	36	7.2±3.7	180±12
E_2-2*	4	68±10	45	9.0±2.0	150±6
E_2-3	5	66±8.0	39	7.9±5.6	190±9
E_2-4	3	82±6.0	20	3.5±1.1	134±5
E_2-5	6	61±5.6	63	12.6±3.0	193±12
E_2-6	4	73±3.0	54	10.8±4.9	189±5

: [a]Doubling time (DT) in hours, was determined as described in (43). DT was significantly different by Student's t-test between BP and all the other cells lines(p<0.001). [b]Colony number (CN); [c]Colony efficiency (CE), and [d]Colony size (CS). These three parameters were significantly different between MCF-10F and all other cell lines (P=0.00001). CS of DES clones was significantly different from E_2 and BP cells (p=0.001). [e]From 24 colonies derived from E2-treated cells, clones E_2-1, E_2-2, E_2-3, E_2-4, E_2-5 and E_2-6 were recovered and expanded. *These cells have been used for detection o microsatellite DNA polymorphism.

Among 67 markers tested, which were selected based on chromosomal changes reported to be present in breast and other cancers, only clones E_2-1 and E_2-2, exhibited LOH in chromosome 11 (Table 2). Clones E_2-1 and E_2-2 identically expressed LOH in chromosome 11 at 11q23.3 (marker D11S29), and 11q24.2-q25 (marker D11S912). BP-treated cells did not exhibit LOH at any of the loci tested. Interestingly, we have found that all the clones of the cells transformed with either E2, BP presented microsatellite instability (MSI), expressed as an allelic expansion at 3p21 locus (marker D3S1447) (data not shown). In order to determine whether these MSIs were related to alterations in mismatch repair genes, we performed microsatellite DNA analysis in loci 1p13.1, with marker BAT40, 2p16, with marker D2S123, and 18q22.3-23, with marker D18S58, which are related to mismatch repair genes. However, none of those markers showed alterations with this technique (Table 2).

TABLE 2: MICROSATELLITE DNA POLYMORPHISM ANALYSIS OF MCF-10F CELLS TREATED WITH 17B-ESTRADIOL (E2) OR BENZ(A)PYRENE (BP)

Ch	Marker	Location	MCF10-F	E2-1	E2-2	BP
1	D1S104	1p21-1p23	O	O	O	O
1	BAT-40	1p13.1	O	O	O	O
2	D2S171	2p24-21	O	O	O	O
2	D2S123	2p16	O	O	O	O
3	D3S1297		O	O	O	O
3	D3S1560	3p26-3p25	O	O	O	O
3	D3S1304	3p26-3p25	O	O	O	O
3	D3S1307	3p26-p25	O	O	O	O
3	D3S1289	3p23-3p21	O	O	O	O
3	D3S1449	3p22.3-3p21.3	O	O	O	O
3	D3S1478	3p21.3-21.2	O	O	O	O
3	D3S2384	3p21.3-21.2	O	O	O	O
3	D3S1450	3p21.1-3p14.2	O	O	O	O
3	D3S1217	3p21	O	O	O	O
3	D3S1447	3p21	O	O	O	O
3	D3S1241	3p21	O	O	O	O
3	D3S1448	3p21	O	O	O	O
3	D3S1480	3p14	O	O	O	O
6	ESR	6q24-27	O	O	O	O
8	MYCL-1	8q24.1	O	O	O	O
9	D9S199	9p23	O	O	O	O
9	D9S157	9p23-22	O	O	O	O
9	D9S171	9p21	O	O	O	O
9	D9S165	9p21	O	O	O	O
11	D11S988	1pter-qter	O	O	O	O
11	D11S922	11p15.5	O	O	O	O
11	H-RAS1	11p15.5	O	O	O	O
11	CCKBR	11p15.4	O	O	O	O
11	D11S1392	11p13	O	O	O	O
11	Int-2	11q13	O	O	O	O
11	D11S907	11p13	O	O	O	O
11	D11S911	11q13-11p23	O	O	O	O
11	D11S436	11p12-11p11.1	O	O	O	O
11	D11S614	11q22-11q23	O	O	O	O
11	D11S940	11q22	O	O	O	O
11	DRD2	11q23.1	O	O	O	O
11	D11S968	11q23.1-11q25	O	O	O	O
11	D11S29	11q23.3	O	•	•	O
11	D11S925	11q23.3-11q24	O	O	O	O
11	D11S912	11q24.2-11q25	O	•	•	O
12	IGF-1	12q22-12q23	O	O	O	O
13	D13S289	13q12.2	O	O	O	O
13	D13S260	13q12.3	O	O	O	O
13	D13S267	13q12.3	O	O	O	O
13	D13S171	13q12.3-13	O	O	O	O
13	D13S218	13q13-14.1	O	O	O	O
13	GABRB-1	13q14.2	O	O	O	O
13	D13S155	13q14.3-21.2	O	O	O	O
16	D16S540		O	O	O	O
17	D17S849	17p13.3	O	O	O	O
17	D17S796	17p13.1	O	O	O	O
17	D17S513	17p13.1	O	O	O	O
17	Tp53	17p13.1	O	O	O	O
17	D17S786	17p13.1	O	O	O	O
17	D17S793	17p13.1-7p11.2	O	O	O	O
17	D17S945	17p13-12	O	O	O	O
17	D17S520	17p12	O	O	O	O
17	D17S800	17q11.1-12	O	O	O	O
17	THRA-1	17q11.2-12	O	O	O	O
17	D17S787	17q21-22	O	O	O	O
17	D17S855	17q21.2	O	O	O	O
17	D17S1323	17q21.2	O	O	O	O
17	D17S808	17q23.2	O	O	O	O

17	D17S789	17q24	O	O	O	O
17	D17S515	17q24.2-25.2	O	O	O	O
17	D17S785	17q25.2	O	O	O	O
18	D18S58	18q22.3-23	O	O	O	O

To obtain DNA, treated and control cells were lysed in 5ml of TNE (0.5M Tris pH 8.9, 10mM NaCl, 15-mM EDTA) with 500μg/ml proteinase K and 1% sodium dodecyl sulfate (SDS), and incubated at 48°C for 24 hours. Following two extractions with phenol (equilibrated with 0.1 M Tris pH 8.0), the DNA was spooled from 2 volumes of 100% ethanol, air dried and resuspended in 20mM EDTA. The DNA was then treated sequentially with RNase A (100 μg/ml) for 1 hour at 37°C and 100μg/ml proteinase K, 1%SDS, at 48°C for 3h, followed by two extractions with saturated phenol. The DNA was again retrieved from the aqueous phase by ethanol precipitation, washed extensively in 70% ethanol, and after air-drying suspended in TE (10mM Tris, pH8.0), 1mM EDTA.

We evaluated for allelic losses the regions of chromosomes 1, 2, 3, 6, 8, 9, 11, 12, 13, 16, 17, and 18 most frequently reported to exhibit loss of heterozygosity (LOH) in spontaneous breast tumors (Table 1). DNA amplification of microsatellite length polymorphisms was utilized for detecting allelic losses present in the transformed clones. Microsatellites are polymorphic markers used primarily for gene mapping which can be broadly defined as relatively short (<100bp) runs of tandem repeated di- to tetranucleotide sequence motifs [61-63]. The origin and nature of these polymorphism sequences is not well established, but they may result from errors of the polymerase during replication and/or from slightly unequal recombination between homologous chromatids during meiosis. These microsatellites have proven to be useful markers for investigating LOH and could be applicable to allelotyping as well as regional mapping of deletions in specific chromosomal regions. They are highly polymorphic, very common (between 10^5 and 10^6 per genome), and are flanked by unique sequences that can serve as primers for polymerase chain reaction (PCR) amplification [64].

Before performing DNA amplification of microsatellite DNA polymorphisms to detect allelic losses present in E_2-, DES-, and BP-treated cells, we verified by DNA fingerprinting whether all the clones derived from MCF-10F treated cells were from the same lineage. Genomic DNA was extracted from the cells listed in Table 1. The identity of these cells was confirmed by Southern blot hybridization of genomic DNA with a cocktail of the three minisatellite probes D2S44, D14S13 and D17S74. Genomic DNAs were digested with HifI, and hybridized with probes under standard condition [64].

Primers used for the analysis of microsatellite polymorphisms are given elsewhere [64]. Conditions for PCR amplification were as follows: 30ng of genomic DNA, 100 pmoles of each oligonucleotide primer, 1 xPCR buffer (Perkin Elmer Cetus), 5μM each of TTP, dCTP, dGTP, and dATP, 1μCi [^{32}P] dATP (300mCi/mmole) (Dupont, NEN, Boston, MA), and 0.5 units of Amplitaq DNA polymerase (Perkin Elmer Cetus) in 10ml volumes. The reactions were processed through 27 cycles of 1 min at 94°C, 1 min at the appropriate annealing temperatures determined for each set of primers, and 1 min at 72°C; with a final extension of 7 min at 72°C. Reaction products were diluted 1:2 in loading buffer (90% formamide, l0mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol), heated at 90°C for 5 min and loaded (4ml) onto 5% to 6% denaturing polyacrylamide gels. After electrophoresis, gels were dried at 70°C and exposed to XAR-5 film with a Lightning Plus intensifying screen at –80°C for 12 to 24 hours. Allele sizes were determined by comparison to M13mp18 sequencing ladders.

LOH was defined as a total loss of, or a 50%, or more reduction in density in one of the heterozygous alleles. All experiments were repeated at least three times to avoid false positive or false negative results. To control for possible DNA degradation, the same blots used to assess allelic loss were analyzed with additional DNA gene probes that detect large fragments. The bands were quantitated using a UltraScan XL laser densitometry (Pharmacia LKB Biotechnology Inc.) within the linear range of the film.

iv Data interpretation

Altogether these data clearly indicate that HBEC when treated with 17-β estradiol produces significant morphogenetic changes. The fact that the MCF10F cells are both ERα and β negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Through this pathway estrogen and estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis [18-23].]. Although this pathway has not been demonstrated in the present work, the data are supporting but not definitively demonstrating the pathway. More studies in this subject are in progress in our laboratory to define this mechanism.

It was of great interest that by the fourth passage after 4 treatments during a two-week period, clones derived from E_2-transformed cells exhibited loss of heterozygosity in chromosome 11, whereas during the same period of time the chemical carcinogen BP did not induce genomic changes, even though we have previously reported that this carcinogen induces LOH in

chromosome 17 [43], in addition to tumorigenesis in a heterologous host after a larger number of passages and a more prolonged selection process *in vitro* [44,45]. We have found that estrogen induces LOH in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2-25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice [65]. Several genes, such as HRAs, CTSD, ILK, TSG101 and KI1 have been reported to be located on the short arm of chromosome 11 [53-54, 65-71]. A region of deletion on 11q22-23 has been described on the long arm of chromosome 11in 40 to 60% of breast tumors [51, 57, 59, 60, 72-74]. The ataxia telangiectasia susceptibility gene (ATM) is the most widely studied candidate gene in this region [75]. ATM may act upstream of the TP53 gene in cell cycle regulation [76,77] and its heterozygous mutation is associated with high incidence of early-onset breast cancer. This region has been reported to contain several tumor suppressor genes and genes involved in the metastatic process. In this latter group, the MMP genes encoding matrix metalloproteases involved in invasion, ETS1 encoding a transcription factor involved in angiogenesis, and VACM-1, encoding a protein probably involved in cell cycle regulation have been identified [78]. Although some of these genes might be affected during the transformation of HBEC induced by estrogens, a more detailed allelotyping using multiple markers is required for better defining the significance of LOH in these cells.

Approximately 35% of breast cancers show LOH at the D11S29 and NCAM loci [79], and a higher frequency of LOH at this locus has also been found in melanomas [80]. LOH has been found at frequencies of 25% and 29% at the distal D11S968 (11qter) and D11S29 (11q23.3 locus), slightly above the accepted baseline of 0-20 per cent in colorectal cancer. The fact that breast cancer, melanoma, and colorectal cancer have been found to be influenced by estrogens [81], give relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23-qter occurs frequently in ovarian and other cancers [82,83].

The most frequent allelic loss observed in breast cancer has been reported in chromosome 17p, suggesting that genes located in that chromosome arm, such as p53 oncogene, might be a likely target for this event. [33, 80-100]. We have not been able up to now to demonstrate any LOH in chromosome 17 in estrogen transformed MCF-10F cells. However, we have used a small number of markers, and the possibility that LOH might be located at sites not tested yet cannot be ruled out. Therefore, the study of allelic imbalances at 17q and 17p, as well as in chromosome 16 [85,101,102] in estrogen transformed HBEC must be carried out to provide further understanding of the functional involvement of these chromosomes in the process of cell transformation by E_2.

The observations that E_2, and BP induce similar phenotypes, but different genomic alterations requires further investigation in order to elucidate the significance of timing of appearance of each type of changes with regards to cancer initiation and progression. There are several probable avenues for explaining these discrepancies. In this model, both estrogens and the chemical carcinogen as an early event induces phenotypic changes, whereas LOH is a rare event that is manifested in different chromosomes and only in few clones derived from E_2 treated cells. The rarity of the phenomenon is in agreement with the low frequency of LOH observed in BP transformed cells, in which the phenomenon is manifested at a more advanced stage of neoplastic progression [43, 100]. Altogether these observations suggest that these two compounds might act through different genetic events for inducing similar transformation phenotypes.

7-KEY RESEARCH ACCOMPLISHMENTS

a- In the present work we have capitalized on the availability in our laboratory of an *in vitro* model of transformation of immortalized HBEC by the chemical carcinogen BP for comparison with phenotypic and genomic changes induced by the natural estrogen 17β-estradiol (E_2).

b- Short term treatment of these cells with physiological doses of 17-β estradiol induces anchorage independent growth, colony formation in agar methocel, and reduced ductulogenic capacity in collagen gel, all phenotypes whose expression is indicative of neoplastic transformation, and that are induce by BP under the same culture conditions. . The fact that the MCF10F cells are both ER-α and ERβ. negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates.

c- Progesterone was unable to induce significant increase in colony formation, although small colonies less than 60 μm in diameter were observed, whereas none were found in the MCF10F cells treated with DMSO. The ductulogenic pattern was not impaired by progesterone but the luminal size was smaller that those found in the MCF10F cells.

d- We have found that estrogen induces LOH in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2-25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice [65].

8-REPORTABLE OUTCOMES

The following publications have been originated from these studies.

1. Russo, J., Hu, Y.F., Tahin, Q., Mihaila, D., Slater, C., Lareef, M.H. and Russo, I.H. Carcinogenicity of Estrogens in Human breast epithelial cells. APMIS 109:000-0000, 2001.
2. Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., Ao, X., and Russo, I.H. 17 beta estradiol is carcinogenic in human breast epithelial cells. Journal of Steroid Biochemistry and Molecular Biology (In press, 2001).
3. Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., and Russo, I.H. The role of estrogen in human breast cancer: a mechanistic view. In: Menopause Hormones and Cancer (Ed. Neves-e-Castro), Parthenon Publishing, England (In press, 2001).
4. Lareef, M.H., Russo, I.H., Slater, C.M., Rogatko, A., and Russo, J. Estrogen induces transformation phenotypes in the estrogen receptor negative MCF10F cells. Proc. Am. Assoc. Cancer Res. 42:4743a, 2001.

9-CONCLUSIONS

The association found between breast cancer development and prolonged exposure to estrogen suggests that this hormone is of etiologic importance in the causation of this disease. In order to prove this postulate we treated the immortalized human breast epithelial cells (HBEC) MCF-10 F with 17beta-estradiol (E_2) for testing whether they express colony formation in agar methocel, or colony efficiency (CE), and loss of ductulogenesis in collagen matrix, phenotypes also induced by the carcinogen benz(a)pyrene (BP). MCF-10F cells were treated with 0.0, 0.07 nM, 70 nM, or 0.25 mM of E_2 twice a week for two weeks. CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses. Ductulogenesis was 75±4.9 in control cells; it

decreased to 63.7±28.8, 41.3±12.4, and 17.8±5.0 in E_2 treated cells, which also formed solid masses or spherical formations lined by a multilayer epithelium, whose numbers increased from 0 in controls to 18.5±6.7, 107±11.8 and 130±10.0 for each E_2 dose. MCF-10F cells were also treated with 3.7 □M of Progesterone (P)-and the CE was 3.39± 4.05. At difference of E_2, progesterone does not impaired the ductulogenic capacity. Genomic analysis revealed that E_2 treated cells exhibited loss of heterozigosity in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2-25, respectively. These results also indicate that E_2, like the chemical carcinogen BP, induces in HBEC phenotypes indicative of neoplastic transformation.

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